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Differentiation of Avian Pathogenic *Escherichia coli* Strains from Broiler Chickens by Multiplex Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic (RAPD) DNA

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Abstract

We examined 50 *Escherichia coli* (*E. coli*) strains isolated from broiler chickens between January 2013 to March 2014 in order to evaluate the epidemiological prevalence of avian pathogenic *E. coli* (APEC) in Jordan by multiplex PCR and random amplification of polymorphic DNA (RAPD) tests. The multiplex polymerase chain reaction (PCR) which was used as tentative criteria of APEC targets 8 virulence associated genes; enteroaggregative toxin (*ast*A), Type 1 fimbria adhesion (*fimH*), iron-repressible protein (*irp*2), P fimbriae (*pap*C), aerobactin (*iuc*D), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), and colicin V plasmid operon (*cva*/*cvi*) genes. The number of detected genes could be used as a reliable index of their virulence. *E. coli* strains already typed as an APEC always harbor 5 to 8 genes, but non-APEC strains harbor less than 4 genes. Assuming the criteria of an APEC is possession of 5 or more virulence associated genes; we found that all 50 *E. coli* strains were classified as APEC strains. The RAPD analysis showed that the *E. coli* strains could be grouped into 35 of RAPD types by using these two different RAPD primer sets, RAPD analysis primer 4 5'AAGAGCCCGT5', and RAPD analysis primer 6 5'CCCGTCAGCA3'. The current study confirmed the endemic nature of APEC in broiler flocks in Jordan. It is essential that the biosecurity on poultry farms should be improved to prevent the introduction and dissemination of APEC and other agents. Furthermore, farmers need to be educated about the signs, lesions, and the importance of this agent.

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Keywords

Avian Pathogenic *Escherichia coli*, Broiler, Multiplex PCR, Random Amplification of Polymorphic DNA

1. Introduction

Escherichia coli (*E. coli*) is one of the most common and important avian bacterial pathogens and infections caused by *E. coli* are responsible for significant economic losses to the poultry industry. *E. coli* also causes intestinal and extra intestinal diseases in domestic and wild animals which lead to severe economic losses throughout the world [1]. Colisepticemia, coligranuloma (Hjarre's disease), air sac disease, coliform salpingitis, coliform cellulitis, swollen-head syndrome, coliform peritonitis, coliform osteomyelitis/synovitis and coliform omphalitis/yolk sac infection are the different forms of *E. coli* infections in poultry [2]. Avian pathogenic *E. coli* (APEC) strains fall under the category of extraintestinal pathogenic *E. coli*, which are characterized by the possession of virulence factors that enable them to live extraintestinal life [3]-[5]. These virulent factors have been identified [3] [5]-[7]. However, no specific virulence factor that contributes entirely to the pathogenicity of APEC has been discovered [4] [8]. Thus a lack of diagnostic tests to determine degrees of the virulence which are primary pathogens (highly virulent), secondary pathogens (moderately virulent), or nonpathogenic (avirulent) makes it difficult to control colibacillosis [9]. In addition, the lack of diagnostic tests also results in the scarce knowledge about epidemiology of APEC. In Jordan, there is no available information of epidemiology of APEC. Taking these circumstances into consideration, we carried out epidemiological study of APEC in broiler chicken farms using multiplex polymerase chain reaction (PCR) and random amplification of polymorphic DNA (RAPD) tests. Multiplex PCR was reported in a previous study [7] [10] [11] and it was proved that virulent strains which are already typed as an APEC had 5 to 8 virulence-associated genes but avirulent strains which are already typed as non-APEC strains had at most 4 virulence associated genes [7]. The RAPD technique provides a rapid, low cost, simple and powerful tool to study avian *E. coli* infections [12] [13].

In this study, we screened 50-field *E. coli* isolated from broiler chickens between January 2013 to March 2014 in order to evaluate the epidemiological prevalence of APEC in Jordan by the multiplex PCR and RAPD tests.

2. Materials and Methods

2.1. Isolation and Identification of Avian E. coli

A total 50 avian *E. coli* strains were isolated from broiler chickens of different ages (hatching up to 5 weeks) in Jordan between January 2013 to March 2014 in Provimi Jordan lab. The *E. coli* strains were recovered from the livers of broilers that had died from colibacillosis. Before death, chickens had shown different signs of *E. coli* infection including septicemia and respiratory infections, with pathological finding, such as air sacculitis, hepatitis, pneumonia, pericarditis, and yolk sac infections. The respective isolates were cultured on 5% horse blood and MacConkey agar. *E. coli* strains were stored in tryptone soy broth (Oxoid, Hampshire, UK) with 15% glycerol at −70˚C.

2.2. DNA Extraction

All *E. coli* strains were cultured on 5% horse blood agar. An agar plate with a pure culture was prepared; and a single colony from each plate was resuspended, washed twice in 150 µl of sterile distilled water, and boiled for 10 min. The suspension was then chilled on ice for 5 min, and the supernatant collected after centrifugation at 13,000 rpm for 5 min in a microcentrifuge (Biofuge; Heraeus Instruments, Langenselbold, Germany).

2.3. Multiplex Polymerase Chain Reaction

All *E. coli* strains were examined for genesenteroaggregative toxin (*ast*A), Type 1 fimbria adhesion (*fimH*), iron- repressible protein (*irp*2), P fimbriae (*pap*C), aerobactin (*iuc*D), temperature-sensitive hemagglutinin (*tsh*), vacuolating autotransporter toxin (*vat*), and colicin V plasmid operon (***cva*/*cvi***) genes by using primer pairs (Al-

pha DNA, Montreal, QC, Canada) previously evaluated in previous studies and are listed in **Table 1**. The multiplex PCR was prepared in a volume of 50 µl as follows: 25 µl of master mix (5 units/µl of Taq polymerase, 400 m*M* deoxynucleotide 5'-triphosphate mixture, and 3 m*M* $MgCl_2$), 18 µl of nuclease-free water (Promega Corp., Madison, WI, USA), 1 µl (50 Pmol/µl) of each primer pair (**Table 1**), and 5 µl of DNA template. Polymerase chain reaction amplification was carried out in a DNA Engine® thermal cycler (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada) for 1 cycle of 3 min at 94˚C, followed by 94˚C for 3 min, 58˚C for 30 sec, and 72˚C for 10 min for 30 cycles. Nuclease-free water used as negative control in each run.

2.4. Random Amplified Polymorphic DNA

Two different primers (Amersham Pharmacia Biotech) previously reported [12] to provide good discriminatory power among avian *E. coil* strains were used, namely, RAPD analysis primer 4 (5'AAGAGCCCGT3') and RAPD analysis primer 6 (5'CCCGTCAGCA3'). The RAPD kit (Amersham Pharmacia Biotech), containing room-temperature stable dried Ready-to-Go beads were used throughout the study. The kit was used as described by the supplier. Briefly as follow, a solution 10 µl of 2.5 Pmol/ml of RAPD analysis reagent ([Ampli*Taq* DNA polymerase and Stoffel fragment], [dNTPs (0.4 m*M* each dNTP in a 25 µl reaction volume], [Bovine serum albumin (2.5 µg)], and [buffer (3 m*M* $MgCl_2$, 30 m*M* KCl and 10 m*M* Tris (pH 8.3)], in a 25 µl reaction volume) was added to each primer, 8 µl of template DNA (10 ng/ml), and 7 µl nuclease-free water (Promega Corp., Madison, WI, USA). The samples were amplified under the following conditions and the reaction was carried out in a DNA Engine® thermal cycler (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada), for 1 cycle of 5 min at 94˚C, followed by 95˚C for 1 min, 36˚C for 1 min, and 72˚C for 2 min for 45 cycles. Visualization of RAPD products were done as above. Nuclease-free water used as negative control in each run.

To confirm the reproducibility of the method, each strain was assayed four times by two different operators who used newly prepared samples and who tested all primers each time. All other experimental conditions remained unchanged, and a result was considered valid when the same pattern was obtained at least three times. The bacterial strains were analyzed with each of the two primers in at least two independent reactions and the banding patterns obtained by at least two runs were considered as a fingerprint for that particular isolate. Different banding profiles were designated by letter codes.

2.5. Agarose Gel Electrophoresis

Polymerase chain reaction products were electrophoresed on a 2% agarose gel in Tris-acetate-EDTA buffer (40 m*M* of Tris and 2 m*M* of EDTA, with a pH value of 8.0) containing ethidium bromide (Promega Corp., Madison, WI, USA) for 1 hour min at 100 V and visualized under UV light (AlphaImager, AlphaInnotech, San Leandro, CA). A 50 bp ladder plus maker (NewEnglands) was used as a molecular weight standard. A digital image of the gel was captured in a computer, and the amplification patterns were evaluated by visual examination of inverted gel pictures.

Table 1. Sequences and specificity of primers, and products size.

No	Virulent Gene	Primer Sequences	Location within Gene	Size (bp)	Primer References
1	*ast*A	5'TGCCATCAACACAGTATATCC3' 5'TCAGGTCGCGAGTGACGGC3'	797 ~ 817 912 ~ 894	116	[31]
2	*vat*	5'TCCTGGGCATAATGGTCAG3' 5'GTGTCAGAACGGAATTGT3'	(−)10 ~ (−)28 282 ~ 264	981	[21]
3	*irp*2	5'AAGGATTCGCTGTTACCGGAC3' 5'AACTCCTGATACAGGTGGC3'	22 ~ 42 434 ~ 416	413	[6] [21]
4	*pap*C	5'TGATATCACGCAGTCAGTAGC3' 5'CCGGCCATATTCACATAA3'	1284 ~ 1304 1784 ~ 1767	501	[32]
5	*iuc*D	5'ACAAAAAGTTCTATCGCTTCC3' 5'CCTGATCCAGATGATGCTC3'	239 ~ 259 952 ~ 934	714	[32]
6	*tsh*	5'ACTATTCTCTGCAGGAAGTC3' 5'CTTCCGATGTTCTGAACGT3'	132 ~ 151 955 ~ 937	824	[21]
7	*Cva*A/B *cvi cva*C	5'TGGTAGAATGTGCCAGAGCAAG3' 5'GAGCTGTTTGTAGCGAAGCC3'	1076 ~ 1095 2056 ~ 2038	1181	[21]
8	*fimH*	5' GGATAAGCCGTGGCCGGTGG 3' 5' CTGCGGTTGTGCCGGAGAGG 3'	215 ~ 235 671 ~ 691	331	[28]

2.6. Analysis of RAPD Data

Each isolate was scored for the presence or absence (1 or 0) of each band on agarose gel. The index of similarity (F) between samples was calculated using the formula [14] $F_{xy} = 2_{nxy}/n_x + n_y$. Where n_{xy} is the number of RAPD bands shared by the two samples and n_x and n_y are the numbers of RAPD bands scored in each sample. The genetic distance (d) was calculated using the formula [15]: $d = 1 - F$. The numerical index of discrimination (D) was calculated using the Simpson's index of diversity [16].

2.7. Statistical Analysis

Association between genes and RAPD patterns were tested by use of the *chi-square* test.

3. Results

3.1. Multiplex PCR

Assuming the criteria of an APEC is possession 5 or more virulence associated genes (Ewers *et al*., 2005); we found that all 50 *E. coli* strains were classified as APEC strains (**Table 2**).There was no strain that contained all 8 of the virulence associated genes. However, there were 4 strains possessed 7 genes (8%), 6 genes were detected in 12 strains (24%), and 5 genes detected in 34 strains (68%). Patterns and combinations of virulence-associated genes for 50 APEC strains isolated in the present study are summarized in **Table 2**. The prevalence of-*fimH* was greatest (94%) among all 8 virulence associated genes, while the lowest (50%) prevalence was noted for *papC* (50%) (**Figure 1**).

3.2. RAPD Analysis of *E. coli* Isolates

Analysis of RAPD banding patterns of 50 isolates revealed a total of 35 distinct patterns (C1 to C35) **Table 2** and **Figure 2**, the 35 RAPD patterns were classified into 5 clusters (A to D). Results were reproducible by repeating the procedure on these isolates on different dates. The discriminating index of two primers as Simpson's index of diversity was calculated 0.965.No association was observed between virulence genespossession and RAPD patterns.

4. Discussion

Previous investigations have indicated that the distribution of various virulence factors are useful markers for the detection and characterization APEC, and could therefore, be used in the diagnosis of colisepticemia in poultry [10] [17]-[19]. The most commonly used molecular genetic finger-printing technique is RFLP analysis. However, RFLP analysis requires relatively large amounts of DNA, expensive equipment and it often takes days to obtain results. By contrast, RAPD results are generated within 4 h and hence are time and cost saving. Several investigators [12] [13] [20] claimed that fingerprinting by RAPD revealed more genetic differences among avian *E. coli* strains than RFLP analysis. In this study, we used a multiplex PCR and RAPD tests to evaluate the epidemiological prevalence of APEC in Jordan.

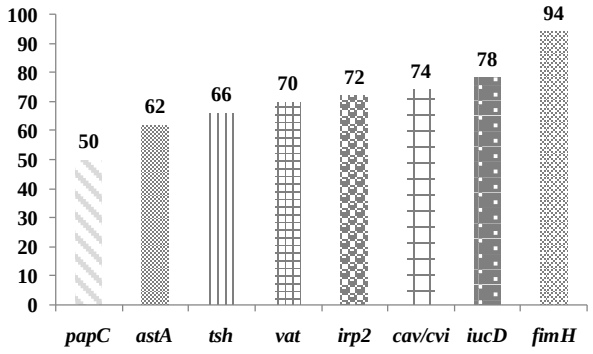

Figure 1. Total detection rates of virulence-associated genes in APEC.

Table 2. Combinations of virulence associated genes and RAPD profiles in 50 isolates of avian *E. coli*.

Strain No	Presences of Virulence-Associated Genes in *E. coli* Strains									RAPD Profiles[1]		Cumulative RAPD[2] Profiles Based on Two Primes
	fimH	*vat*	*tsh*	*iuc*D	*pap*C	*irp*2	*cav/cvi*	*ast*A	Total No of Virulence Associated Genes	Primer 4	Primer 6	
1	+	+	+	+	−	+	+	+	7	A1	B1	C1
2	−	+	+	+	−	+	+	−	5	A1	B1	C1
3	+	+	+	−	−	+	+	−	5	A1	B1	C1
4	+	+	−	+	−	+	−	+	5	A1	B2	C2
5	+	−	+	+	−	−	+	+	6	A2	B3	C3
6	+	+	−	+	−	+	−	+	5	A3	B4	C4
7	+	+	−	+	+	+	+	−	5	A4	B5	C5
8	−	−	+	+	+	+	−	+	5	A5	B6	C6
9	+	−	+	−	−	+	+	+	5	A5	B6	C6
10	+	+	+	+	−	+	+	−	5	A6	B6	C7
11	+	+	+	−	−	+	+	−	5	A7	B7	C8
12	+	+	−	+	−	−	+	+	5	A8	B7	C9
13	+	+	+	−	−	+	+	+	6	A9	B7	C9
14	+	+	−	+	+	+	+	−	6	A9	B8	C10
15	+	+	+	+	−	+	−	+	5	A10	B8	C11
16	+	+	−	+	−	+	−	+	5	A10	B9	C12
17	+	−	+	+	−	+	+	−	5	A11	B10	C13
18	+	+	−	+	−	+	+	−	5	A12	B11	C14
19	+	+	−	+	+	+	+	+	6	A12	B12	C15
20	+	−	+	+	+	−	+	+	5	A12	B13	C16
21	+	+	−	+	−	−	+	+	5	A13	B13	C17
22	+	+	+	−	−	−	+	+	5	A14	B13	C18
23	+	−	+	−	+	+	+	−	5	A15	B14	C19
24	+	+	+	−	+	+	+	−	6	A16	B15	C20
25	+	+	+	+	−	−	+	+	5	A17	B15	C21

Strain No	Presences of Virulence-Associated Genes in *E. coli* Strains[1]									RAPD Profiles[2]		Cumulative RAPD[3] Profiles Based on Two Primes
	fimH	*vat*	*tsh*	*iuc*D	*pap*C	*irp*2	*cav/cvi*	*ast*A	Total No of Virulence Associated Genes	Primer 4	Primer 6	
26	+	+	+	+	−	+	+	+	6	A18	B15	C22
27	+	+	+	+	−	−	+	−	5	A19	B15	C23
28	+	+	+		+	+	+	−	5	A19	B16	C24
29	+	−	+	+	+	−	+	+	5	A19	B16	C24
30	+	+	−	+	+	−	+	+	5	A20	B17	C25
31	−	+	−	+	+	+	+	+	6	A21	B18	C26
32	+	−	−	+	+	+	+	−	5	A22	B18	C27
33	+	+	−	+	+	+	−	+	6	A23	B19	C28
34	+	−	+	+	−	+	−	+	5	A23	B20	C29
35	+	−	+	+	+	−	+	+	5	A23	B20	C29
36	+	+	−	+	+	+	+	−	6	A23	B20	C29
37	+	+	+	+	−	+	+	−	5	A23	B20	C29
38	+	+	+	−	−	+	+	+	6	A23	B20	C29
39	+	−	+	−	+	−	+	+	5	A24	B21	C30
40	+	−	+	+	+	−	+	−	5	A24	B21	C30
41	+	+	+	−	+	+	−	+	6	A25	B22	C31
42	+	+	−	+	−	+	−	+	5	A25	B22	C31
43	+	+	+	+	+	+	−	+	7	A26	B23	C32
44	+	+	+	+	+	−	+	+	7	A27	B24	C33
45	+	−	+	+	+	+	−	+	5	A28	B25	C34
46	+	−	+	+	+	+	+	−	5	A28	B25	C34
47	+	+	+	+	+	+	−	+	7	A28	B26	C35
48	+	−	+	+	−	+	−	+	5	A28	B26	C35
49	+	+	−	+	+	−	+	−	5	A28	B26	C35
50	+	+	−	+	+	+	+	−	6	A28	B26	C35

[1]+ and –, gene detected and undetected, respectively, by PCR. [2]A1-A28 and B1-B26 represent the different RAPD fragment patterns with respect to each primer. [3]C1-C35 represents the different RAPD fragment patterns based on two primers primer.

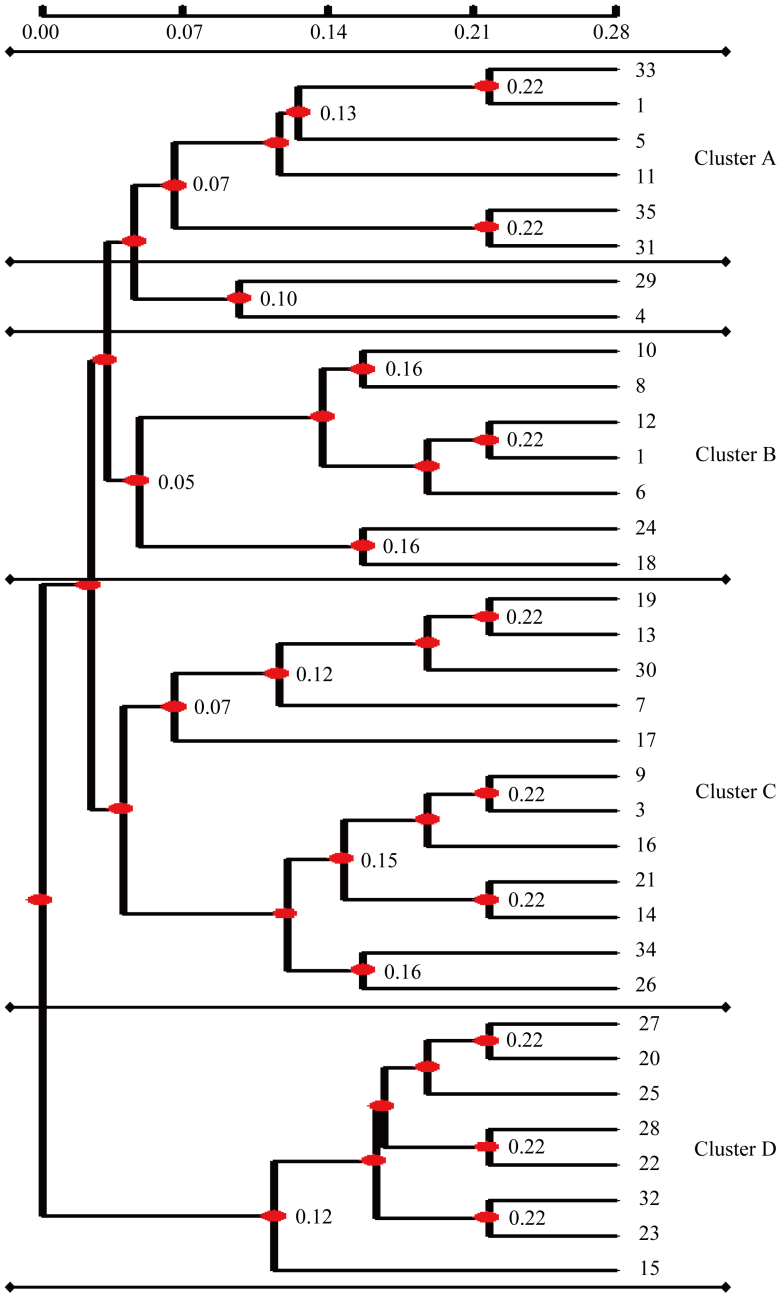

Figure 2. Dendrogram showing the relationships among 50 *E. coli* isolates of 35 RAPD types generated for primers 4 and 6.

We tentatively determined that the criteria of APEC were harboring of more than 5 virulence-associated genes as previously reported [6] [7]. According to the criteria, all *E. coli* examined in this study are classified as APEC (**Table 2**). As we expected, there was no genes which were exclusively detected in APEC and no exact combination which always applies to APEC. However, by means of this study, we detected the pattern of gene combination and estimated the importance of genes among the 8 virulence-associated genes.

The gene *fimH* was predominantly distributed among all genes. The high prevalence of type I fimbriae in this study (94%) is in accordance with published data [21]-[23]. Previous investigators have shown that APEC isolates can bind to avian tracheal cells and type I fimbriae confer this ability [22]. Despite the fact that type I fimbriae are also widely in *E. coli* isolates of mammals [21], they are claimed to be responsible for the first step in

the colonization of the lungs and air sacs of birds. This indicates that *fimH* have important role in the pathogenesis of avian colibacillosis.

The gene*pap*C, indicating the existence of the *papC* operon, was present in 50% of all investigated strains. P-fimbriae are known to adhere to internal organs and to protect the *E. coli* from antibacterial action of neutrophils [22].

Tsh and *vat* genesencodes an auto-transporter protein which shows similarity to a subclass of the IgA protease family [3] [24]. *Tsh* coding for temperature sensitive hemagglutinin and was considered to be involved in the mechanisms of adherence to the avian respiratory tracts [3] [20]. *Tsh* has been identified as an important trait of APEC in several studies [3] [20]. 70% and 66% of investigated strains gave positive results for *vat* and *tsh*, respectively. Herein results confirming broad distribution of this gene among APEC strains.

*Irp*2 and *iuc*D, both related to iron acquisition system [6]. *Iuc*D and *Irp*2 were detected in78 and 72% in tested strains, respectively. Our data indicated that the possession of *Iuc*D and *Irp*2 genes are the specific genetic marker for investigated strains.

*Ast*A was found to be widely distributed among different categories of diarrheagnic *E. coli* in humans and animals, and the toxin was also expressed by 38% of none pathogenic *E. coil* strains [25]. The wide distribution of the gene is attributed to be due to its location on a transposon [25] [26]. *AstA* was detected in 62% of tested strains. Thus, we can only assume that the expression of this toxin gene may be of any relevance in the pathogenesis of colibacillosis.

Herein data also represented the importance of colicin V plasmid operon (*cva*/*cvi*) gene. Colicin Vplasmids were found primarily among virulent enteric bacteria and have been shown to encode several virulence related properties in addition to colicin V [6] [27] [28]. Possessing *iuc*D, *tsh* and *cva*/*cvi*, Colicin Vplasmids have been considered to be a defining feature of the APEC strains [6] [28].

The RAPD analysis results presented here show that the 50 avian *E. coli* strains collected from broiler chickens in Jordan could be differentiated into 35 different RAPD sub-types with five major clusters designed (A-D). No association was observed between virulence genes possession and RAPD patterns.

RAPD results of this study revealed that avian *E. coli* genetically very heterogenic. This result is in agreement with results of Chansiripornchai *et al*. [12] and Maurer *et al*. [20] finding. It is also not uncommon to find more than one *E. coli* genetic type from the same bird. Similar findings have been reported for *E. coli* in cattle [20], *Staphylococcus aureus* in dairy cows [29], and *E. coli* in poultry [30]. Maurer *et al*. [20] encountered certain *E. coli* RAPD types throughout the year [20]. The flocks do not have enough time to develop immunity to the *E. coli* genetic type due to the brief longevity of broiler and this may cause encountering different *E. coli* types.

In RAPD analysis, Maurer *et al*. [20] found 16 different RAPD types in 84% *E. coli* isolates. In thisstudy, RAPD recognized 35 different profiles among 50 *E. coli* isolates. In another study, Chansiripornchai *et al*. [12] found 50 RAPD types by using two different primers in 55 *E. coli* strains. The use of different and more than one RAPD primers may improve differentiation power of RAPD process. Chansiripornchai *et al*. [12] used six different primers in their research. The same researchers found from these primers thatrandom primer number 4 and 6 gave highest discriminatory power on *E. coli* strain from different flock (Chansiripornchai *et al*., 2001). For this reason random primer number 4 and 6 were selected in this study.

5. Conclusion

In conclusion, by utilizing such diagnostic techniques, it is possible to conduct a detailed epidemiological study to determine the full economic effect of colibacillosis. Future research can be done to try and establish an *in vivo* chicken infection model to determine the actual pathogenicity of the strains used in the present study for the correlation of the virulence gene RAPD profiles with pathogenicity in chickens.

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